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Plasmids contribute to food processing environment–associated stress survival in three Listeria monocytogenes ST121, ST8, and ST5 strains

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Abstract

Listeria monocytogenes is a food-borne pathogen responsible for the disease listeriosis and is commonly isolated from food and food production facilities. Many L. monocytogenes strains contain plasmids, though the contributions of plasmids to survival in food production environments is unknown. Three L. monocytogenes ST5, ST8, and ST121 strains containing plasmids, which harbor putative stress response genes, were cured of their plasmids. Wildtype (WT) and plasmid-cured strains were exposed to disinfectant, oxidative, heat, acid, or salt stress. After stress exposure, cells were plated for colony forming unit (CFU) counts to determine survivors. L. monocytogenes WT strains exposed to 0.01% (vol/vol) H2O2, 1% (vol/vol) lactic acid, and 15% (wt/vol) NaCl, pH 5 showed significantly higher counts of survivors compared to the plasmid-cured strains. The number of survivors for the ST5 WT strain exposed to 10 μg/mL benzalkonium chloride (BC) was significantly higher than in the plasmid-cured strain. The ST8 and ST5 strains were exposed to elevated temperature (50° and 55°C respectively); only the ST5 WT strain had significantly higher numbers of survivors than the plasmid-cured strains. Our data revealed that L. monocytogenes ST5, ST8, and ST121 plasmids contribute to tolerance against elevated temperature, salinity, acidic environments, oxidative stress and disinfectants.

Keywords

Listeria monocytogenes, plasmid, oxidative stress, benzalkonium chloride, acidic stress, salt stress

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Plasmids contribute to food processing environment–associated stress survival in three *Listeria monocytogenes* ST121, ST8, and ST5 strains

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Abstract

Listeria monocytogenes is a food-borne pathogen responsible for the disease listeriosis and is commonly isolated from food and food production facilities. Many *L. monocytogenes* strains contain plasmids, though the contributions of plasmids to survival in food production environments is unknown. Three *L. monocytogenes* ST5, ST8, and ST121 strains containing plasmids, which harbor putative stress response genes, were cured of their plasmids. Wildtype (WT) and plasmid-cured strains were exposed to disinfectant, oxidative, heat, acid, or salt stress. After stress exposure, cells were plated for colony forming unit (CFU) counts to determine survivors. *L. monocytogenes* WT strains exposed to 0.01% (vol/vol) H_2O_2, 1% (vol/vol) lactic acid, and 15% (wt/vol) NaCl, pH 5 showed significantly higher counts of survivors compared to the plasmid-cured strains. The number of survivors for the ST5 WT strain exposed to 10 µg/mL benzalkonium chloride (BC) was significantly higher than in the plasmid-cured strain. The ST8 and ST5 strains were exposed to elevated temperature (50° and 55°C respectively); only the ST5 WT strain had significantly higher numbers of survivors than the plasmid-cured strains. Our data revealed that *L. monocytogenes* ST5, ST8, and ST121 plasmids contribute to tolerance against elevated temperature, salinity, acidic environments, oxidative stress and disinfectants.

Key words

Listeria monocytogenes; plasmid; oxidative stress; benzalkonium chloride; acidic stress; salt stress

1. Introduction

In food production facilities, food-borne pathogens are of high concern and can – when consumed - cause a variety of food-borne illnesses, ranging from relatively mild symptoms such as nausea, vomiting, or diarrhea to severe symptoms; some foodborne illnesses can be life-threatening (Kaur et al., 2007; Mead et al., 1999; Scallan et al., 2011). Food-borne pathogens are constantly exposed to environmental stresses during food processing, including high or low temperatures, high salinity, acidity/alkalinity and low nutrient availability, all of which temper their ability to survive (Gandhi and Chikindas, 2007; Larsen et al., 2014; Leistner and Gorris, 1995; Schirmer et al., 2014). Furthermore, the routine cleaning and disinfection procedures in food processing environments (FPEs) provide additional challenges for microorganisms such as detergents and disinfectants resulting in toxic conditions for bacterial growth and survival (Barker et al., 2003; Ratani et al., 2012). Food-borne pathogens are also exposed to food-specific stress conditions such as varying levels of salinity or acidity due to an assortment of compounds, such as acetic acid, lactic acid, sodium chloride, calcium chloride, or other fermentation products (Davies et al., 1997; Gahan et al., 1996; Luna-Guzman and Barrett, 2000; O'Driscoll et al., 1996). Among food-borne pathogens, *L. monocytogenes* is of particular concern due to its presence in ready-to-eat food, its capability to survive in food and FPEs, and the high mortality of listeriosis (Allerberger and Wagner, 2010). *L. monocytogenes* is known to colonize niche areas such as drainage and hard-to-clean surfaces, which allow the bacteria to survive or even proliferate and thus, making it difficult to completely eradicate from FPEs (Ferreira et al., 2014).

Persistence, as used in this article, is defined as the repeated occurrence of genetically indistinguishable *L. monocytogenes* strains in the same food production facility over a long period of time (Carpentier and Cerf, 2011; Ferreira et al., 2014). Furthermore, identical strains may be

found in the same time period but in unrelated facilities, indicating that persistence of *L. monocytogenes* is not necessarily plant specific (Autio et al., 2002).

The *L. monocytogenes* strain classification via multi-locus sequence types (ST) is widely used for characterization of strains from clinical samples, food or FPEs. In-depth epidemiological studies have identified STs that are found predominantly in human clinical samples, while other STs have been shown to be particularly abundant in food and FPEs (Maury et al., 2016). The *L. monocytogenes* sequence types ST121, ST5 and ST8, can be highly abundant in food and FPE, and therefore exposed to variable stress conditions (Cruz et al., 2014; Fagerlund et al., 2016; Henri et al., 2016; Knudsen et al., 2017; Martin et al., 2014; Maury et al., 2016). *L. monocytogenes* strains isolated from food and FPEs are often found to contain plasmids. Plasmid frequencies ranged from 28% to 81% and plasmids sizes ranged from 14 to 106 kbp (Fagerlund et al., 2016; Harvey and Gilmour, 2001; Hingston et al., 2017; Kolstad et al., 1992; Kuenne et al., 2010; Lebrun et al., 1992; McLauchlin et al., 1997; Rychli et al., 2017; Schmitz-Esser, Muller, et al., 2015). Some *Listeria* plasmids can be identical and can be recovered from strains over multiple years and from different geographic locations, indicating a high level of conservation of some *Listeria* plasmids (Fox et al., 2016; Kuenne et al., 2010; Muhterem-Uyar et al., 2018; Rychli et al., 2017; Schmitz-Esser, Muller, et al., 2015). However, a lower level of conservation of plasmids was found e.g. in ST8 strains (Fagerlund et al., 2016). Additionally, *L. monocytogenes* plasmids exhibit a modular genetic structure (Canchaya et al., 2010; Kuenne et al., 2010), though the function of most of these plasmid genes has not yet been identified. Some functional data on the effect of plasmids on stress survival has been described recently, such as their contribution to heat stress survival, heavy metal, antibiotics, or benzalkonium chloride tolerance (Elhanafi et al., 2010; Kremer et al., 2017; Lebrun et al., 1994; Li et al., 2016; Pöntinen et al., 2017).

In general, plasmids can be easily lost as they impose a maintenance cost and are only retained if they confer an evolutionary or selective advantage for survival. *L. monocytogenes* strains isolated from food production facilities over several years were found to retain their plasmids, an indication for a benefit for the retention of these plasmids that compensates for their maintenance cost (Ferreira et al., 2014; Knudsen et al., 2017; Kuenne et al., 2010; Martin et al., 2014).

We hypothesize that these plasmids confer an advantage to survival when *L. monocytogenes* strains are exposed to stress conditions found in food and FPEs. To examine this, we used three sets of isogenic *L. monocytogenes* strains belonging to ST5, ST8 and ST121, each consisting of a pair of plasmid-harboring wildtype and plasmid-cured strains to determine the effect of plasmids on stress survival. The plasmids were carefully analyzed with regard to their gene contents and retained after exposure to a variety of stress conditions. We reveal a higher stress tolerance among strains containing plasmids, thereby showing that plasmids confer advantages for survival in stress environments that *L. monocytogenes* is exposed to in food and FPEs.

2. Materials and methods

2.1. Bacterial strains, growth conditions, plasmid curing and plasmid screening

Plasmids were cured from three *L. monocytogene*s ST121, ST8, and ST5 strains (Table 1). These strains have been identified as persistent in food and FPEs previously, see Table 1, and have been used as models for studying various aspects of survival of *L. monocytogenes* in a number of studies (Casey et al., 2014; Fagerlund et al., 2016; Fox et al., 2016; Harter et al., 2017; Muhterem-Uyar et al., 2018; Müller et al., 2013; Müller et al., 2014; Rychli et al., 2017). For plasmid curing, strains were grown overnight at 37°C in tryptic soy broth and yeast extract (TSB+y) in a shaking

incubator at 100 rpm. 100 µL of the overnight culture was pipetted into 10 mL of TSB+y that contained sub inhibitory concentrations (0.2 and 0.3 µg/mL) of novobiocin (Oxoid). The cultures inoculated with novobiocin were then exposed to increased heat, 40°C overnight, with shaking at 125 rpm. Exposure to both elevated temperature and the novobiocin should result in curing of the plasmid. Diluted cultures of 10^{-1} to 10^{-4} were then streaked on tryptic soy agar (TSA, BD) or ALOA plates (Chromogenic Listeria Agar, Oxoid) and incubated overnight at 37°C. Single colonies were screened for the presence of plasmids with PCR using primers targeting a 600 bp region of the plasmid replication protein gene *rep*A (Forward: 5' – CGCCGTTTTTGATCACTGTA-3; Reverse: 5'-AGCAAGTACCAATCGGAAGG-3'; T_A: 62°C). Primers were designed using Primer3 (Untergasser et al., 2012). In a PCR final volume of 50 µL, the following concentrations were used: 10 µM of each primer, 50 mM $MgCl_2$, 10 mM dNTP mix, buffer, 1.5 U Platinum Taq polymerase (Life Technologies), and DEPC-treated water. PCR cycle conditions were as follows: initial denaturation for 3 minutes at 94°C, 35 cycles of denaturation at 94°C for 30 seconds, annealing at 62°C for 30 seconds and elongation at 72°C for 30 seconds, final elongation at 72°C for 4 minutes. Genomic DNA was isolated from all three sets of isogenic *L. monocytogenes* strain pairs for use as positive controls. Negative controls with no template and positive controls were used in all PCR reactions. All PCR products were confirmed with agarose gel electrophoresis. After confirmation of curing, wildtype and cured strains were routinely grown overnight on TSA or in TSB at 20°C.

2.2. Stress survival assays

For all stress survival assays, each strain was inoculated into 5 mL of TSB (Fisher Chemical) and grown overnight at 20°C with shaking at 200 rpm. Overnight cultures were adjusted

to a starting inoculum of an optical density (OD) at 600nm of 0.2 in TSB. 100 µL of starting inoculum was used to inoculate 5 mL of TSB and exposed to the stress conditions as described below. The exposure time for all stress conditions was two hours. Experimental cultures were conducted in triplicate for each experiment and placed in a shaking incubator at 200 rpm at 20°C for 2 hours. A temperature of 20°C was chosen to provide conditions similar room temperature. Tenfold serial dilutions of the WT and plasmid-cured cultures after stress exposure were plated on TSA plates with duplicates for each concentration to determine the difference in CFUs between the WT and plasmid-cured strains. Determination of CFUs was conducted after 24 hours of incubation of the plates at 37°C. Log_{10} reduction values were calculated based on the obtained CFU counts using the following formula: Log_{10} reduction = mean log_{10} (CFUs WT) – mean log_{10} (CFUs plasmid-cured).

Experimental culture media and strains varied depending on the stress conditions tested: The wildtype strains 6179, R479a, and 4KSM, and their plasmid-cured derivatives were subjected to an oxidative stress, acidic stress, and salt stress survival test. For the oxidative stress test, a final concentration of 0.01% (vol/vol) H_2O_2 was applied. For acidic stress survival, TSB, with a final concentration of lactic acid of 1% (vol/vol), pH 3.4 was used. To test for combined salt and mild acidic stress survival, experimental cultures were inoculated into TSB, with 15% (wt/vol) NaCl, adjusted to a pH of 5 with hydrochloric acid. For each strain (6179, R479a, 4KSM; for both wildtype and plasmid-cured strains), experiments with each of the three different stress conditions (15% NaCl, pH 5; 0.01% H_2O_2; 1% lactic acid, pH 3.4) were conducted in triplicate.

Testing for benzalkonium chloride survival was conducted with the WT and the plasmid-cured strain of 4KSM only, as only 4KSM contains the *bcrABC* cassette, which confers increased tolerance to BC, on the plasmid p4KSM, whereas 6179 contains Tn*6188*, a chromosomally

encoded BC resistance marker (Müller et al., 2013). The R479a WT strain is naturally sensitive to BC (Müller et al., 2013) and thus was not tested for BC tolerance here. For the BC stress test, each strain was inoculated into TSB with a final concentration of 10 µg/mL BC.

The ST5 and ST8 strains were also analyzed in a heat stress assay. As pLM6179 contains an identical copy of the *clpL* gene found in pLM58, recently shown to be responsible for heat stress tolerance (Pöntinen et al., 2017), we did not test heat stress with 6179. Maximum growth temperatures were determined prior to survival experiments, indicating a maximum growth temperature of 50°C for R479a, and 55°C for 4KSM. Initial overnight setup and OD measurements for the heat stress assay were identical to the other stress assays. Cultures were inoculated into TSB prewarmed to 50° or 55°C and placed in a shaking incubator at 50° or 55°C for 2 hours. All serial dilutions, plating, and CFU counts were conducted identically to the previous stress assays.

2.3. Sequence analysis

Sequence analysis including annotation and comparative BLAST searches of the plasmids was performed in PATRIC (Wattam et al., 2017). The average nucleotide identity between the plasmids was determined using the JSpecies webserver (Richter et al., 2016). Phylogenetic analyses of plasmid replication initiation protein RepA amino acid sequences was performed with MEGA7 using maximum likelihood based phylogenetic inference and the JTT amino acid substitution model with 1000x bootstrapping (Kumar et al., 2016).

2.4. Statistical analysis

Statistical analysis was conducted on Microsoft Office Excel 2013. Differences in CFU counts between WT and plasmid-cured strains were tested using student paired two-tailed t-test. Graphing was conducted in JMP Pro 14, with standard error used for calculation of error bars.

3. Results

3.1. Gene content of the analyzed plasmids

To determine the overall relatedness of the plasmids, we analyzed the average nucleotide identity (ANI) between the three plasmids. pLM6179 and pLMR479a shared a higher ANI than they did with the ST5 plasmid p4KSM. Although the ANI values were above 95%, the overall overlap between the plasmids was lower than 17%, except for pLM6179 and pLMR479a, with more than 52% coverage (Table S1, Fig. S1). Similarly, pLM6179 and pLMR479a showed higher average amino acid identity between shared plasmid proteins compared to p4KSM (Table S2). pLM6179 and pLMR479a shared 46 proteins. p4KSM and pLM6179 shared 26 proteins, whereas p4KSM and pLMR479a shared 40 proteins. 22 proteins where shared among all three plasmids (Fig. S2).

We analyzed the gene content of the three plasmids from the ST121, ST5, and ST8 strains to determine putative plasmid genes that may contribute to stress survival (Table 2). Many of the plasmid genes cannot be assigned with a putative function, but for some, a possible function can be deduced. Furthermore, there were some genes that we identified on all three plasmids. All three strains used for our experiments contain the transposon Tn*5422* on their plasmids, which is involved in cadmium tolerance (Lebrun et al., 1994). However, while the pLM6179 and pLMR479a Tn*5422* *cadA* and *cadC* genes share 100% amino acid identity, the p4KSM Tn*5422* shows 69% and 55% amino acid identity with the pLM6197 and pLMR479a homologs,

respectively. All three plasmids contain a RepA plasmid replication protein. We found that the pLM6179 RepA shared 99% amino acid identity with the RepA protein on p4KSM. The pLMR479a RepA protein shared 97% amino acid identity with the p4KSM and pLM6179 RepA proteins. Phylogenetic analyses of RepA amino acid sequences revealed that all three plasmids belonged to group 2 *Listeria* plasmids established by (Kuenne et al., 2010) (Fig. S3).

The plasmid of ST121 *L. monocytogenes* strain 6179 contains a ClpL protein, a member of the HSP100 subgroup of heatshock proteins. The *clpL* gene found on pLM6179 is identical to the *clpL* gene found in the plasmid pLM58, which has been shown to be responsible for increased heat stress survival in *L. monocytogenes* (Pöntinen et al., 2017).

The *L. monocytogenes* ST8 strain R479a plasmid genes possibly involved in stress survival include a putative multicopper oxidase and copper transporter, which have been shown in *Staphylococcus aureus* to be involved in copper homeostasis and oxidative stress response (Kosman, 2010; Ladomersky and Petris, 2015; Sitthisak et al., 2005). The *Staphylococcus aureus* and R479a proteins share 95% and 100% amino acid identity with the oxidase and copper transporter, respectively. Two putative heavy metal transporting ATPase genes that may be involved in resistance to heavy/transition metals are present on pLMR479a (Lewinson et al., 2009). pLMR479a also contains a putative NADH peroxidase, Npx, which has been shown to act as a hydrogen peroxide reducer in *Lactobacillus casei*, thus decreasing oxidative stress (Gibson et al., 2000; La Carbona et al., 2007; Serata et al., 2012). Additionally, a putative glycine betaine transport binding protein, GbuC, is present on pLMR479a, which may reduce the effect of osmotic stress (Angelidis and Smith, 2003; Ko and Smith, 1999).

One notable locus on the *L. monocytogenes* ST5 strain 4KSM plasmid is the *bcrABC* cassette that conveys increased tolerance to quaternary ammonium compounds and was first

characterized on pLM80 (Elhanafi et al., 2010). The p4KSM BcrABC copy shares 100% amino acid identity with BcrABC from pLM80. The 4KSM plasmid also contains a putative ClpB protein, a distant homolog to the ClpL heatshock protein found in pLM6179 and pLM58. The p4KSM Clp protein belongs to the ClpA/B family (InterPro domain: IPR001270). Similar to pLMR479a, a putative heavy metal transporting ATPase, a NADH peroxidase Npx, as well as a GbuC protein are found on p4KSM. p4KSM also contains an identical homolog of the triphenylmethane reductase characterized from the *L. monocytogenes* plasmid pLM80 which has been shown to be involved in crystal violet detoxification (Dutta et al., 2014).

3.2. Survival of wildtype strains compared to plasmid-cured strains without stress conditions.

Our study is based upon the premise that plasmids aid in survival under stress conditions found in food and food production facilities. To more accurately reflect temperatures found in such conditions, our studies were conducted at 20°C and not at 37°C. To ensure that the plasmid curing had no detrimental effect on growth under regular growth conditions without stress, we analyzed the growth difference of the WT and plasmid-cured derivatives of the *L. monocytogenes* 6179, R479a, and 4KSM strains at 20°C with no stressors added. Although the CFUs were numerically lower for the plasmid-cured strains, we found no significant effect upon growth differences due to the temperature or the removal of the plasmid from the WT strains (Fig. S4).

3.3. Survival of wildtype strains compared to plasmid-cured strains exposed to stress conditions

In this study, we analyzed and compared the stress survival between *L. monocytogenes* WT and plasmid-cured strains when exposed to environmental stress typically found in food and food production facilities. In order to observe the effect of plasmids on oxidative stress survival, we compared the stress survival between the *L. monocytogenes* WT strains 6179, R479a, and 4KSM and their plasmid-cured derivatives when exposed to sublethal concentrations of H_2O_2 for two hours. All three of the WT strains tested were significantly more tolerant to oxidative stress in comparison to their plasmid-cured derivatives ($P < 0.001$) (Fig. 1 A). Curing of the plasmids resulted in 0.06 (pLM6179), 0.2 (p4KSM) and 0.42 (pLMR479a) $\log_{10}$ reduction of the survivors.

As bacteria are often exposed to various acidic stress in food and food production facilities, *L. monocytogenes* WT and plasmid-cured strains were exposed to a pH of 3.4, adjusted with lactic acid. Our results show that all three WT strains tested were also significantly more tolerant to acidic stress in comparison to their plasmid-cured derivatives ($P < 0.001$) (Fig. 1 B). Plasmid-cured strains exhibited a 0.08 (pLM6179), 0.18 (p4KSM) and 0.26 (pLMR479a) $\log_{10}$ reduction of the survivors.

Acidity and high salinity are frequent co-stressors observed e.g. in meat and dairy production facilities and fermented foods. Therefore, we compared the stress survival response between the *L. monocytogenes* strains 6179, R479a, and 4KSM, both WT and the plasmid-cured derivatives, when exposed to high salinity and low pH (pH 5). We found *L. monocytogenes* WT strains 6179 and 4KSM to be more tolerant in comparison to the plasmid-cured strains ($P < 0.05$). A much stronger contribution of plasmids to the same stress conditions was found for R479a ($P < 0.001$) (Fig. 1 C). Absence of the plasmids resulted in 0.06 (pLM6179), 0.13 (p4KSM) and 0.18 (pLMR479a) $\log_{10}$ reduction of the survivors.

We analyzed and compared the stress survival between the 4KSM WT and plasmid-cured strains, when exposed to sublethal concentrations of BC, a quaternary ammonium compound often found in industrial disinfectants. We anticipated an effect on stress survival for the 4KSM WT strain over the plasmid-cured derivative as p4KSM encodes a *bcrABC* cassette that provides increased tolerance to BC (Elhanafi et al., 2010). This assumption was confirmed as, after two hour stress exposure period, the *L. monocytogenes* 4KSM WT strain proved to be significantly more tolerant to BC in comparison to the plasmid-cured strain ($P < 0.001$) when exposed to sublethal concentrations of BC (Fig. 2) resulting in a 0.17 $\log_{10}$ reduction.

In addition to cold temperatures, *L. monocytogenes* can be exposed to heat stress during food production in FPEs. Due to the presence of a Clp-like protein on the p4KSM, we anticipated an increased tolerance to heat stress in the WT strain over the plasmid-cured. We found that the 4KSM WT strain was significantly more tolerant to heat stress when exposed to 55°C for two hours in comparison to the plasmid-cured strain ($P <0.001$) (Fig. 3). Since we did not identify a putative protein that may mediate heat stress response in the R479a plasmid we hypothesized no effect for this isolate. Consistently, the R479a WT strain showed no significant difference in tolerance to heat stress when compared to the plasmid-cured strain when exposed to 50°C for two hours ($P = 0.74$) (Fig. 3).

4. Discussion

Listeria monocytogenes is a major contributor to global foodborne illnesses and foodborne deaths and is capable of long term survival in food and FPEs and its ability to adapt to stresses contributes to the persistent nature of the pathogen. Moreover, it has been noted that while the *L. monocytogenes* core genome is highly stable, there are a variety of mobile genetic elements

interspersed (den Bakker et al., 2010; Kuenne et al., 2013), and some of them contribute to stress response and environmental adaptation (Dutta et al., 2013; Harter et al., 2017; Müller et al., 2013; Orsi et al., 2008; Ryan et al., 2010; Verghese et al., 2011). However, little data is available on the functional characteristics of *L. monocytogenes* plasmids in regards to stress survival mechanisms in food and FPEs. Therefore, we examined the differences between WT and plasmid-cured strains exposed to stress conditions that may be found in such conditions. Experimental data on *Listeria* plasmids has almost exclusively been focused primarily on antibiotic, heavy metal and disinfectant resistance (Elhanafi et al., 2010; Kremer et al., 2017; Lebrun et al., 1994; Li et al., 2016; Pöntinen et al., 2017). A recent study provided preliminary indirect evidence for a contribution of *L. monocytogenes* plasmids to acid tolerance but did not analyze this in more detail using e.g. plasmid-cured strains and/or identification of the responsible plasmid genes (Hingston et al., 2017). Here, we perform a broad analysis comparing three distinct plasmids from strains from three different *L. monocytogenes* STs under various stress conditions.

Our results show that plasmids in the analyzed *L. monocytogenes* ST5, ST8, and ST121 strains confer increased tolerance under stress conditions relevant for food and FPE. More specifically, we show that p4KSM provides increased tolerance to heat stress at 55°C. Recently, pLM58 from an ST9 *L. monocytogenes* strain has been described to confer heat stress tolerance at 55°C through a ClpL protein encoded on pLM58 (Pöntinen et al., 2017). The ClpL from pLM58 is identical to the ClpL found on pLM6179, strongly suggesting that the ClpL in pLM6179 does also provide increased heat stress tolerance. p4KSM does not encode a ClpL protein, but a ClpB-like protein, which is only distantly related to the pLM58 ClpL (29% amino acid identity, 38% coverage). The p4KSM ClpB-like protein is also significantly shorter than the pLM58 ClpL: 704 amino acid residues compared to 372 amino acid residues for the ClpB-like protein in p4KSM.

Highly similar or identical proteins are found in numerous *Listeria* other *Firmicutes* genomes, all with highly similar predicted lengths, thus suggesting that the 4KSM Clp protein is most likely not a truncated pseudogene. Clp proteins have been described to be involved in various stress response reactions (Chastanet et al., 2004; Tao and Biswas, 2013); a contribution of the p4KSM Clp protein to heat stress response might be conceivable. No significant differences in heat stress tolerance at 50°C were found for the R479a WT and plasmid-cured strains. Thus, pLMR479a does not seem to provide heat stress tolerance. In line with this, and in contrast to p4KSM and pLM6179, based on sequence analysis, no proteins were identified on pLMR479a that may confer heat stress tolerance.

p4KSM also confers increased tolerance to BC which is most likely encoded by the *bcrABC* cassette present on p4KSM. The p4KSM BcrABC proteins share 100% amino acid identity with BcrABC from pLM80 which have been demonstrated to provide increased tolerance to BC (Elhanafi et al., 2010). The efflux transporter proteins BcrBC from p4KSM also share 53% and 55% amino acid identity with the EmrC protein on pLMST6 which also confers BC tolerance (Kremer et al., 2017). In this context, it is interesting to note that p4KSM derives from a strain that was isolated from a food production facility with a long-term *L. monocytogenes* contamination. The ST5 strains (including 4KSM) persisted in this plant for several years and became the dominant ST in this facility, in spite of massive usage of various disinfectants including BC-based disinfectants. Interestingly, none of the initially abundant *L. monocytogenes* strains in this plant harbored a plasmid and after three years, the two remaining abundant STs (ST5 and ST204) from this facility harbored identical plasmids. Thus, plasmids have been suggested to be important for the persistence of ST5 strains in this FPE based on sequence analyses (Muhterem-Uyar et al.,

336 2018). Here, we provide experimental evidence for the contribution of plasmids in 4KSM, an ST5
337 strain from this FPE, to survival under stress conditions.

338 All three plasmids analyzed here significantly increased the tolerance of the strains to
339 0.01% hydrogen peroxide, 1% lactic acid, pH 3.4 and 15% sodium chloride, pH 5 (Fig. 1). For
340 hydrogen peroxide, p4KSM and pLMR479a encode candidate genes which might be responsible
341 for mediating the oxidative stress response. p4KSM and pLMR479a encode an identical putative
342 NADH peroxidase, both show 45% amino acid identity to homologs in *Enterococcus faecalis*
343 which are responsible for oxidative stress response (La Carbona et al., 2007). Other candidate
344 genes possibly involved in oxidative stress response on pLMR479a include a putative multicopper
345 oxidase, a Dps family protein, and YbbML homologs which have been shown to be involved in
346 oxidative stress response in other bacteria (Nicolaou et al., 2013; Olsen et al., 2005; Sitthisak et
347 al., 2005; Tu et al., 2012).

348 All three plasmids contributed significantly to tolerance to 1% lactic acid, pH 3.4. Similar
349 to the results from oxidative stress response, pLMR479a and p4KSM showed a higher contribution
350 to stress response compared to pLM6179. Based on the annotation and predicted functions of the
351 pLM6179 proteins, no candidate gene which might mediate acid stress response could be
352 identified.

353 All three plasmids were also involved in response to mild acidic stress combined with high
354 osmotic stress (15% sodium chloride, pH 5). While pLM6719 and p4kSM contributed to a lesser,
355 still significant, degree to stress response, the contribution of pLMR479a was much higher. R479a
356 was isolated from smoked salmon and persisted in this fish processing facility for several years
357 (Fonnesbech Vogel et al., 2001). Thus, the higher salt tolerance conferred by pLMR479a might
358 reflect an adaptation to high salt concentrations commonly found in fish processing and smoking.

359 Based on the predicted functions of the pLM6179 proteins, no candidate genes which might be
360 responsible in mild acid stress and high osmotic stress response could be identified. The plasmids
361 p4KSM and pLMR479a contain identical putative GbuC proteins which are glycine/betaine
362 binding proteins of the *L. monocytogenes* GbuABC transporter and have been shown to be
363 involved in osmotic stress response (Angelidis and Smith, 2003). However, p4KSM and
364 pLMR479a contain only a GbuC homolog, no GbuA or GbuB homologs are found on the plasmids.
365 The benefit of the additional GbuC homologs may thus be in conjunction with other
366 chromosomally encoded osmotic stress response proteins such as the chromosomally encoded
367 GbuABC proteins, which are present in the R479a and the 4KSM chromosomes (data not shown).
368 As shown for the first time on phenotypic and genomic level, plasmids pLM6179,
369 pLMR479a and p4KSM have a clear survival-triggering function in the *L. monocytogenes* isolates
370 to stresses that can be encountered in many FPEs. For all stress conditions analyzed, the
371 highlighted proteins potentially involved in mediating stress response are candidates only. It is
372 possible that other plasmid proteins are responsible for conferring the observed stress responses;
373 these might include many of the uncharacterized plasmids proteins for which we cannot assign a
374 putative function based on sequence annotation and similarity. Further functional characterization
375 such as gene expression data, expression of proteins in other hosts, or deletion mutants of candidate
376 genes will be required to identify which plasmid proteins are involved in response to certain stress
377 conditions.
378
379 **5. Conclusion**
380 Taken together, our study provides evidence that the analyzed plasmids provide increased
381 tolerance against different stress conditions including disinfectants, heat, high salt concentrations

382 combined with mild acid stress, and acid stress. Our results thus broaden our knowledge about the
383 function of *L. monocytogenes* plasmids in stress response and show that *L. monocytogenes*
384 plasmids can aid in the response to different stress conditions. Based on the reported high
385 conservation of some, or large parts of, *L. monocytogenes* plasmids, the results of our study could
386 thus potentially also apply to other *L. monocytogenes* plasmids.
387
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395
396

Figure Legends

Figure 1. Survival of wildtype (WT) and plasmid-cured *L. monocytogenes* strains under different stress conditions at 20°C, displayed as $\log_{10}$ CFU/ml values and $\log_{10}$ reduction values based on CFU/ml. To display $\log_{10}$ reduction values, the CFU/ml values for the wildtype strains were set as zero. Error bars show standard deviation among three replicate data sets. Stress conditions were: A) 0.01% H_2O_2; B) 1% lactic acid, pH 3.4; C) 15% NaCl, pH 5. Stress exposure time was two hours for all conditions. P-values are as follows: *: $P \leq 0.05$, ***: $P \leq 0.001$.

Figure 2. Survival of wildtype (WT) and plasmid-cured *L. monocytogenes* ST5 strain 4KSM exposed to 10 µg/mL of benzalkonium chloride for two hours displayed as $\log_{10}$ CFU/ml values and $\log_{10}$ reduction values based on CFU/ml. To display $\log_{10}$ reduction values, the CFU/ml values for the wildtype strain were set as zero. Error bars show standard deviation among three replicate data sets. P-values are as follows: ***: $P \leq 0.001$.

Figure 3. Survival of wildtype (WT) and plasmid-cured *L. monocytogenes* ST5 strain 4KSM exposed to heat stress at 55°C for 2 hours, and WT and plasmid-cured ST8 strain R479a to heat stress at 50°C for 2 hours. Values are displayed as $\log_{10}$ CFU/ml and $\log_{10}$ reduction values based on CFU/ml. To display $\log_{10}$ reduction values, the CFU/ml values for the wildtype strain were set as zero. Error bars show standard deviation among three data sets. P-values as follows: ***: $P \leq 0.001$, NS: No significant difference between the WT and plasmid-cured strains.

420 **Table 1. *Listeria monocytogenes* wildtype strains harboring plasmids used in this study.**

Strain	Sequence Type	Country of origin	Isolation source, year	Plasmid	Plasmid size (kbp)	GenBank accession number	Reference
6179	ST121	Ireland	Cheese, 2000	pLM6179	62.2	HG813250	(Schmitz-Esser, Muller, et al., 2015)
R479a	ST8	Denmark	Smoked Salmon, 1996	pLMR479a	86.6	HG813248	(Schmitz-Esser, Gram, et al., 2015)
4KSM	ST5	Austria	Food production environment, 2011	p4KSM	90.5*	JYOJ01000032.1*, JYOJ01000033.1*	(Muhterem-Uyar et al., 2018)

421 * p4KSM has not been closed and consists of 2 contigs.

422

423

Table 2. Presence and conservation of plasmid encoded candidate genes involved in stress survival.

Plasmid	Tn*5422* (CadA)	Tn*5422* (CadC)	BcrABC	ClpL	ClpB	NADH Peroxidase	Triphenylmethane reductase	Multicopper Oxidase	GbuC
pLM6179	+ *(100%)	+ *(100%)	-	+	-	-	-	-	-
pLMR479a	+ *(100%)	+ *(100%)	-	-	-	+ *(100%)	-	+	+ *(100%)
p4KSM	+ *(69%)	+ *(55%)	+	-	+	+ *(100%)	+	-	+ *(100%)

* (amino acid identity)

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Fig. 1

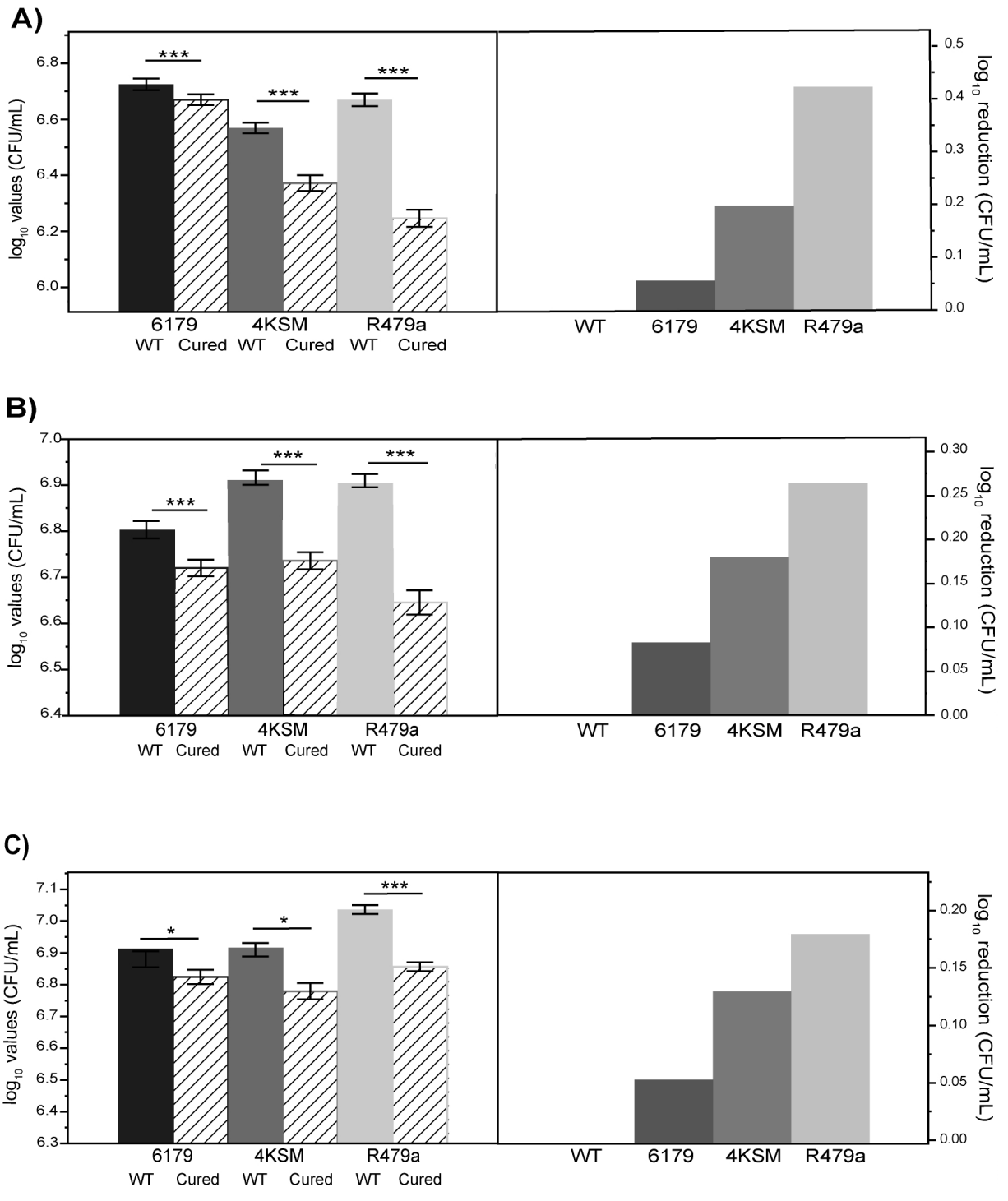
A)
log10 values (CFU/mL)
6.8
6.6
6.4
6.2
6.0
***
***
***
6179
WT Cured
4KSM
WT Cured
R479a
WT Cured
log10 reduction (CFU/mL)
0.5
0.4
0.3
0.2
0.1
0.0
WT
6179
4KSM
R479a
B)
log10 values (CFU/mL)
7.0
6.9
6.8
6.7
6.6
6.5
6.4
***
***
***
6179
WT Cured
4KSM
WT Cured
R479a
WT Cured
log10 reduction (CFU/mL)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
WT
6179
4KSM
R479a
C)
log10 values (CFU/mL)
7.1
7.0
6.9
6.8
6.7
6.6
6.5
6.4
6.3
*
*
***
6179
WT Cured
4KSM
WT Cured
R479a
WT Cured
log10 reduction (CFU/mL)
0.20
0.15
0.10
0.05
0.00
WT
6179
4KSM
R479a

Fig. 2

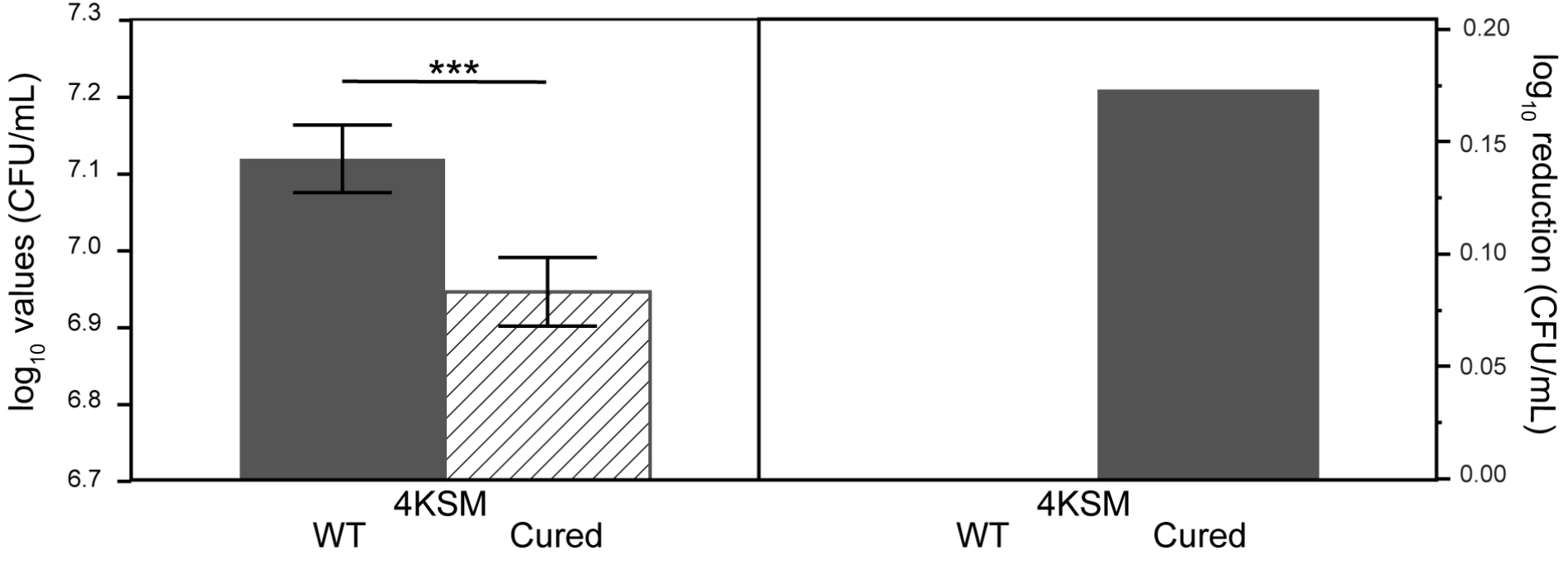
7.3
7.2
7.1
7.0
6.9
6.8
6.7
log10 values (CFU/mL)
***
0.20
0.15
0.10
0.05
0.00
log10 reduction (CFU/mL)
4KSM
WT
Cured
4KSM
WT
Cured

Fig. 3

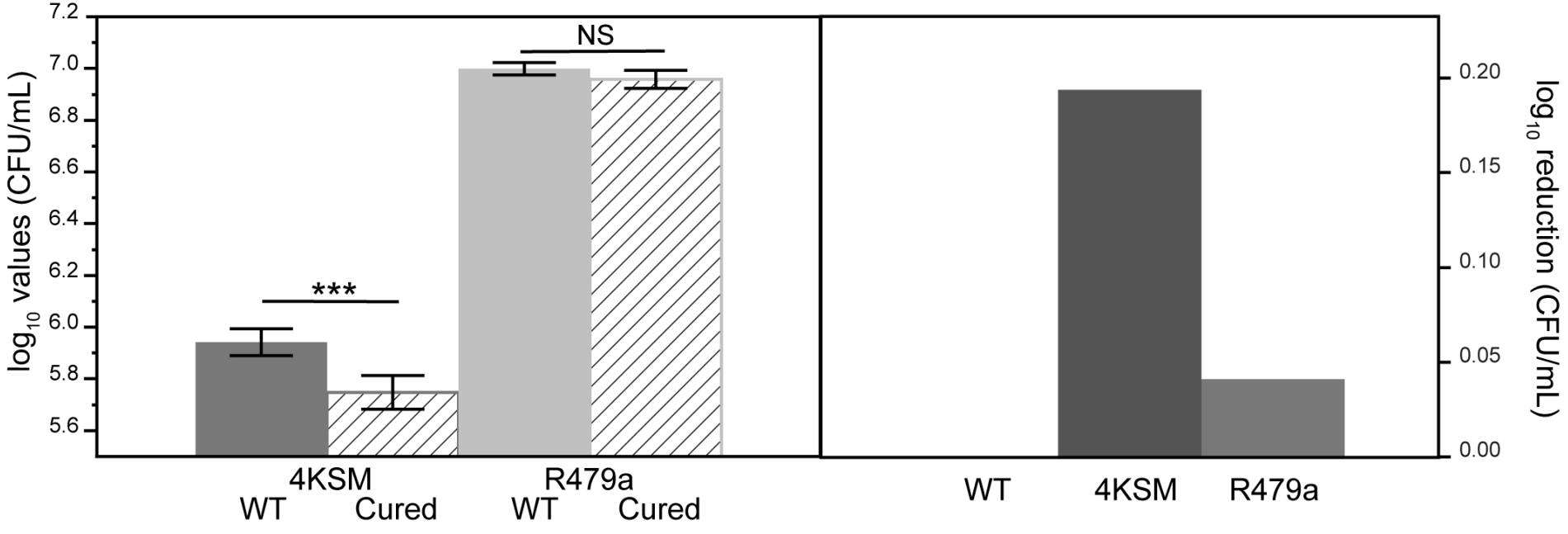
7.2
7.0
6.8
6.6
6.4
6.2
6.0
5.8
5.6
log10 values (CFU/mL)
NS
***
4KSM
WT
Cured
R479a
WT
Cured
0.20
0.15
0.10
0.05
0.00
log10 reduction (CFU/mL)
WT
4KSM
R479a

Table S1: Average Nucleotide Identity (ANI) among the pLM6179, pLMR479a, and p4KSM plasmids. ANI was determined with the JSpecies webserver and the mummer algorithm. The ANI is shown as percent identity. The alignment coverage is shown in parentheses.

	pLM6179	**pLMR479a**	**p4KSM**
pLM6179	-	96.98% (73.11)	95.90% (9.45)
pLMR479a	96.98% (52.51)	-	96.35% (17.51)
p4KSM	95.90% (6.49)	96.35% (17.21)	-

Table S2: Average amino acid identity between shared plasmid proteins. Average amino acid percent identity was determined with a BLAST comparison between the pLM6179, pLMR479a, and p4KSM plasmids with PATRIC.

	pLM6179	**pLMR479a**	**p4KSM**
pLM6179	-	94.8%	47.8%
pLMR479a	90.0%	-	58.9%
p4KSM	47.9%	60.7%	-

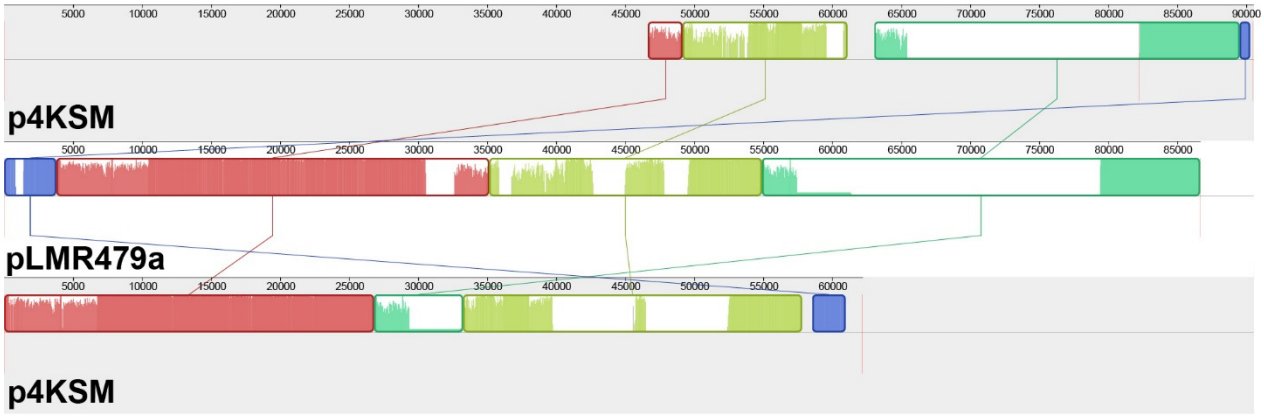

Figure S1: Alignment of p4KSM, pLMR479a, and pLM6179 plasmids. The plasmids were aligned with MAUVE (Darling et al, 2010, PLoS One;5(6):e11147.). Homologous regions have the same color and the height of the blocks correlates with the conservation level of the regions for each plasmid.

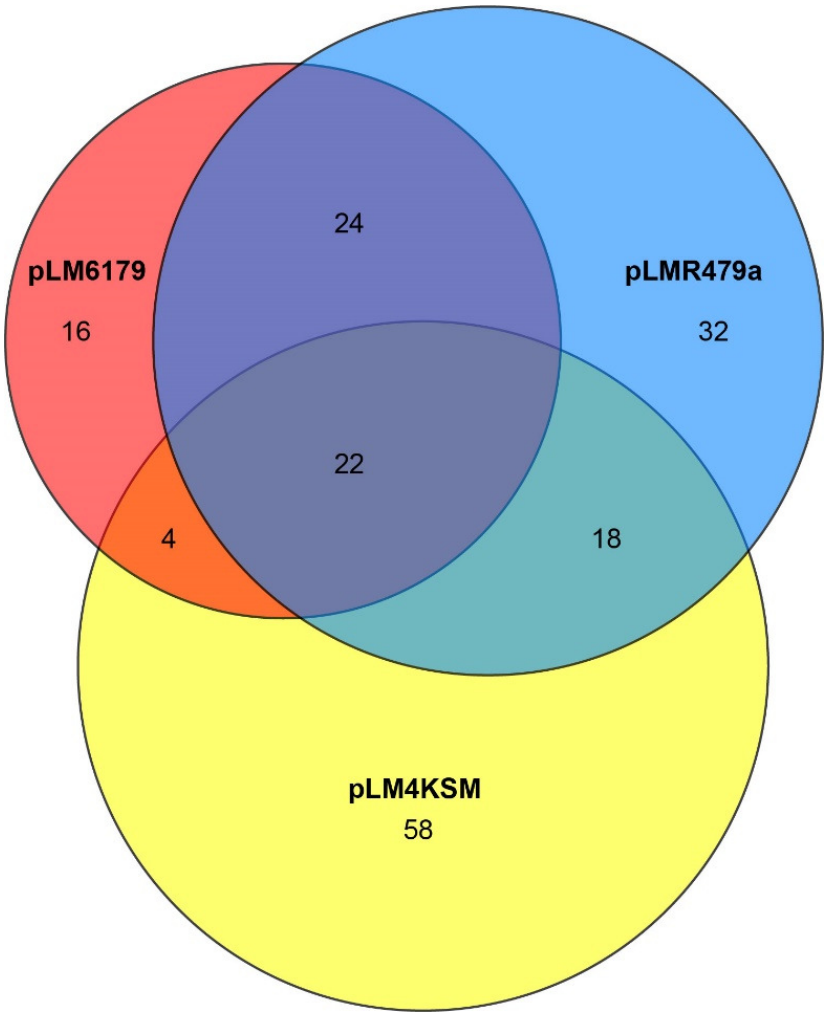

Figure S2. Venn diagram showing shared proteins between pLM6179, pLMR479a, and p4KSM. The number of proteins as either shared or unique is shown within the diagram. Numbers in the overlapped areas indicate the number of shared proteins between the plasmids. The central number in the figure is the number of proteins shared by all three plasmids.

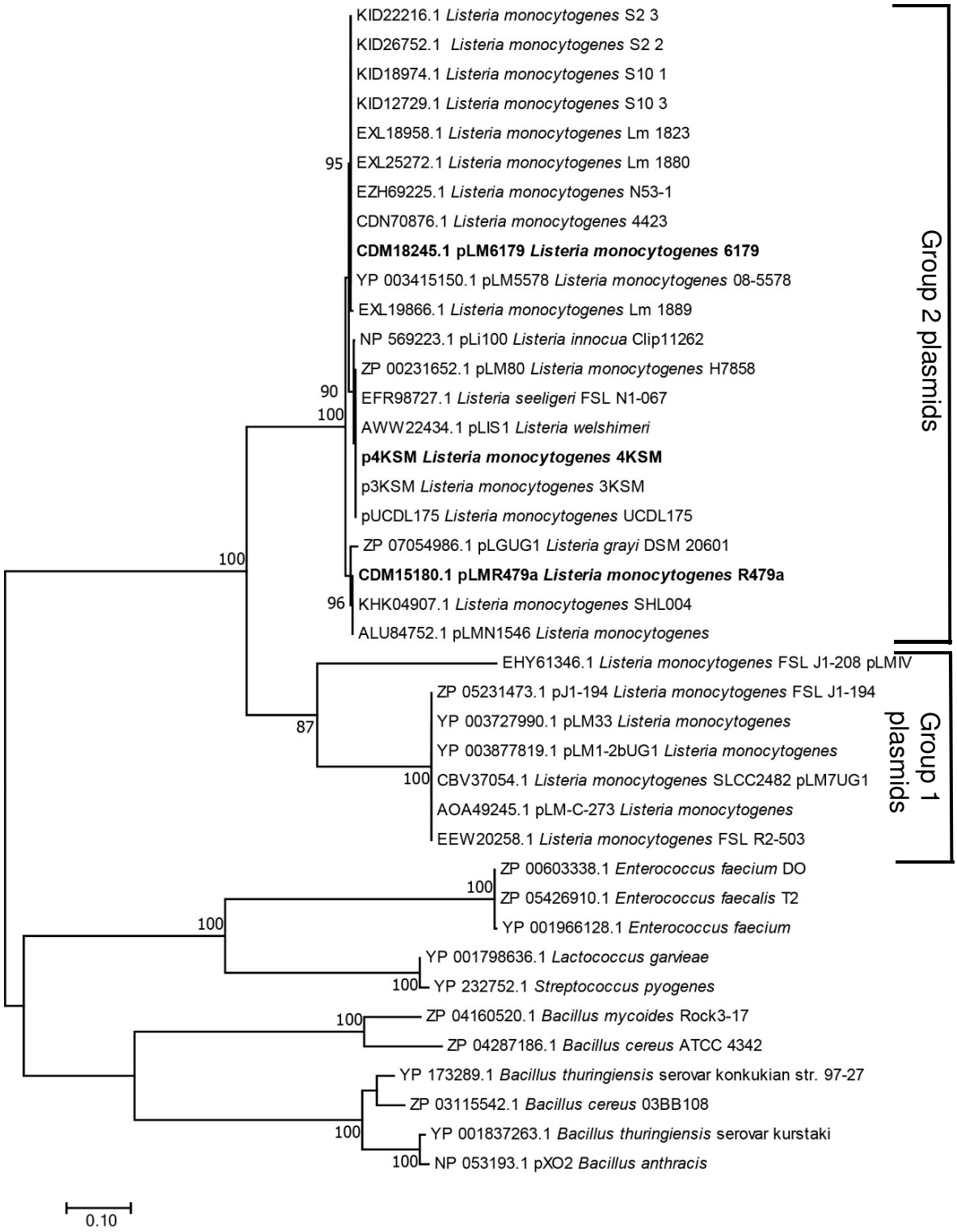

Figure S3: Phylogenetic relationships of *Listeria* plasmids The evolutionary history based on RepA replication initiation proteins amino acid sequences was inferred by using the Maximum Likelihood method based on the JTT matrix-based model. The tree with the highest log likelihood (-5786.66) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 40 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 370 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar S., Stecher G., and Tamura K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Molecular Biology and Evolution 33:1870-1874.).

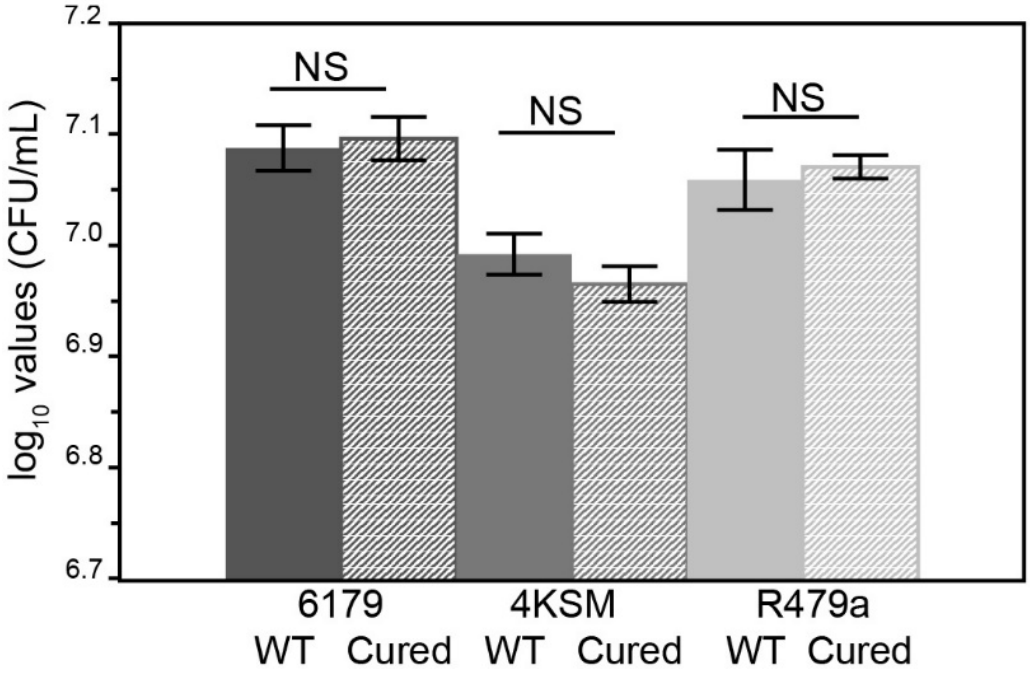

Figure S4. Growth of wildtype (WT) and plasmid-cured *L. monocytogenes* strains without stress conditions at 20℃ determined using CFU/ml. Error bars show standard deviation among three data sets. NS indicates no significant difference ($p > 0.05$) between the WT and plasmid-cured strains.